Administration of adenoviral vectors induces gangrene in acutely ischemic rat hindlimbs: Role of capsid protein-induced inflammation

Lucy S. Brevetti, MD, Rajabrata Sarkar, MD, PhD, David S. Chang, MD, Madison Ma, BS, Robert Paek, MD, and **Louis M. Messina, MD,** *San Francisco, Calif*

Purpose: **The initial purpose of this study was to determine the effects of intravascular adenoviral vector-mediated gene transfer of endothelial nitric oxide synthase (AdeNOS) on experimental hindlimb ischemia in the rat. Unexpectedly, administration of AdeNOS immediately after induction of acute limb ischemia led to limb gangrene. We subsequently sought to define the molecular mechanisms responsible for this unusual effect and to devise adenoviral gene transfer strategies to prevent the development of gangrene in acutely ischemic limbs.**

Methods: **Phosphate-buffered saline or adenoviral vectors containing the bovine endothelial nitric oxide synthase gene (AdeNOS) or no transgene (Ad-E1) were injected intra-arterially into the hindlimb of a rat under vascular isolation immediately after surgical induction of severe ischemia. Hematoxylin and eosin staining was performed on muscle sections to evaluate inflammation. A separate group of animals was injected with an adenovirus containing a nontranscribable genome, treated with cyclosporine, or received delayed administration of the adenoviral vector. Gene expression after delayed adenoviral gene transfer was assessed with immunohistochemistry, Western blotting, and nitric oxide synthase (NOS) activity assay.**

Results: **Both AdeNOS and Ad-E1 caused gangrene of the entire hindlimb within 12 days in a dose-dependent manner, at a threshold concentration of 1×10^9 plaque-forming unit/mL. Adenoviral delivery was associated with more inflammation and edema compared with phosphate-buffered saline histologically. Inactivation of adenoviral DNA transcription did not affect induction of gangrene. However, gangrene was prevented by concurrent immunosuppression with cyclosporine or delayed administration of the vector. Delayed administration allowed adenoviral gene expression as determined by immunohistochemistry, NOS protein levels, and an assay of NOS enzyme activity.**

Conclusion: **Intra-arterial administration of adenoviral vectors, under vascular isolation, immediately after induction of acute ischemia causes inflammation and subsequent limb gangrene. The inflammatory response is unrelated to the expression of the recombinant transgene or the adenoviral genome and is likely due to the adenoviral capsid proteins. However, administration of cyclosporine or delayed injection of the adenoviral vector is a method that can be used for adenoviral mediated gene transfer in limb ischemia. (J Vasc Surg 2001;34:489-96.)**

Currently, adenoviral vectors are the most commonly used vectors for direct in vivo gene transfer to a variety of tissues and experimental models of disease. The advantages of recombinant adenoviral vectors include the ability to infect a wide variety of nonreplicating cells within brief exposure periods, the potential for delivery of large recombinant proteins that cannot be packaged in retroviral vectors, and high levels of recombinant protein expression. Efficient transduction with adenoviral vectors can be achieved with 15 to 30 minutes of exposure, which is comparable to the cross-clamp time required for many vascular surgical procedures. Also, the ability to infect nonreplicating cells is critical for gene transfer to endothelial and smooth muscle cells in vivo. These features make adenoviral vectors particularly suitable for gene transfer to the blood vessels.

Adenoviral gene transfer to blood vessels has been used to determine the in vivo function of particular proteins[1] and to inhibit neointimal hyperplasia in experimental models of restenosis.[2] Although adenoviral gene transfer to the vasculature has focused on large conduit arteries and experimental models of diseases that affect such vessels, prior studies from our laboratory have focused on gene transfer to the limb capillary bed and skeletal muscle fibers. We have shown that intra-arterial administration of adenoviral vector into a rat hindlimb under vascular isolation for 30 minutes results in biologically active levels of recombinant protein, which could be detected both locally and systemically.[3] The use of adenoviral vectors in the capillary bed of ischemic limbs could potentially provide high-level expression of proteins tar-

From the Department of Surgery, Division of Vascular Surgery, University of California.

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Reprint requests: Louis M. Messina, MD, University of California San Francisco M-488, 505 Parnassus Avenue, San Francisco, CA 94143-0222 (e-mail: Messina@surgery.ucsf.edu).

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geted to treatment of various aspects of limb ischemia. Directed expression of recombinant angiogenic proteins in the capillary bed could treat ischemia[4] and could also be used to express proteins that prevent small vessel thrombosis or promote tissue regeneration.

A detrimental feature in the use of adenoviral vectors in vivo, particularly in vascular applications, has been a local inflammatory reaction induced by the virus. Although initial reports in which recombinant adenoviral vectors were used to transduce the arterial wall did not identify a significant inflammatory reaction,[5,6] subsequent studies in other models have shown significant vessel wall inflammation after the administration of recombinant adenovirus to large vessels in vivo.[1,7,8]

Whereas our earlier studies demonstrate significant gene expression in nonischemic hindlimbs,[3] the effects of adenoviral gene transfer to the capillary bed of an ischemic limb remain unknown. Our preliminary studies demonstrated that the intra-arterial administration of adenoviral vectors in the setting of severe acute limb ischemia surprisingly resulted in frank gangrene of the entire limb. The purpose of this study was to characterize the mechanisms responsible for this clinically significant phenomenon and to devise experimental strategies that would allow adenoviral gene transfer in ischemic limbs.

MATERIALS AND METHODS

Adenoviral vectors. All adenoviral vectors contained deletions of E1 and E3 regions and were replication defective. Adenoviral vector-mediated gene transfer of endothelial nitric oxide synthase (AdeNOS) contains the bovine endothelial nitric oxide synthase (eNOS) complementary DNA driven by the Rous sarcoma virus promoter and was a generous gift of Dr Beverly L. Davidson (University of Iowa College of Medicine).[9] Ad-E1, which is an identical vector in all respects to AdeNOS yet does not contain a transgene, was used as a control. Propagation of vectors was performed as described.[3] After two rounds of viral purification with cesium chloride, viral stocks were dialyzed twice against 3% sucrose and stored in 3% sucrose at –80°C. Lack of replication competent virions was confirmed by a cytopathic effect (CPE) assay on HeLa cells. In brief, HeLa cells at 10^6 per well in a six-well plate were inoculated with the adenoviral vector at a multiplicity of infection of 20 to 40 in 1 mL of media for 2 hours in humidified 5% carbon dioxide atmosphere at 37°C. The media were replaced and the cells incubated and monitored daily for CPE. The cells were split 1:4 every 3 days for 2 weeks without any signs of CPE. For animal experiments, frozen vector stocks were thawed immediately before use and were diluted in sterile phosphate-buffered saline (PBS) to achieve a final concentrations ranging from 1×10^8 to 1×10^{10} plaque-forming unit (pfu)/mL. The concentrations of all adenoviral vectors were assayed with the optical absorbance assay and the standard plaque assay.[10] The optical absorbance is a physical assay performed to measure the concentration of the adenoviral particles. Briefly, a sample of the solution with the virions is placed in a cuvette, and the optical density at 260 nm (OD260) is measured. The concentration of virions is calculated according to the data of Maizel et al.[11] The plaque assay is performed to measure the concentration of biologically infectious virions. In brief, 293 cells plated at 10^6 per well in a six-well plate were incubated for 16 to 24 hours in humidified 5% carbon dioxide atmosphere at 37°C. The cells were inoculated with the adenoviral vector at serial dilutions for 90 minutes. The vector-containing medium was gently aspirated and discarded. The cells were overlaid with minimal essential medium containing 15% fetal bovine serum, 4 mmol/L glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 1% SeaPlaque low-melting point agarose. Titer plates were then cultured in a humidified, 5% carbon dioxide atmosphere at 37°C. Plaques were counted on day 14 by visual inspection. The mean of the number of plaques from three separate wells, each inoculated with 100 μL of stock vector from dilution, was determined, and the titer was calculated by multiplying this value by the dilution factor. In our laboratory, the ratio of total viral particles to biologically infectious virions is ranges from 200 to 400:1.

8-Methoxypsoralen inactivation of adenovirus. To determine if the deleterious effects of adenovirus administration were due to the expression of adenoviral proteins from open reading flames present within the vectors, we used 8-methoxypsoralen (8-MOP)–inactivated adenoviral vectors. The 8-MOP is an intercalating agent, which irreversibly prevents DNA transcription. Ultraviolet radiation of 8-MOP–treated adenovirus causes cross-linking of the adenoviral DNA strands, thus preventing its transcription.[12,13] A total of 300 μL per well of Ad-E1 was added to wells of a 24-well tissue culture plate. The 8-MOP (Sigma, Cat No M3501, St Louis, Mo) dissolved in dimethoxysulfoxide was added to each well at a final concentration of 330 μg/mL. The plate was kept on ice and exposed to ultraviolet light (365 nm at a distance of 3 cm) for 30 minutes. Unreacted 8-MOP was removed by means of dialysis against 3% sucrose. The 8-MOP–treated Ad-E1 (8-MOP/E1) was then assayed with two methods: a physical assay, measuring the optical absorbance, and a biological assay, with the standard plaque assay technique as above. The physical assay is performed to measure the concentration of the adenoviral particles, and the biological assay is performed to confirm inactivation of the viral genome.

Animals. A total of 35 male Sprague-Dawley rats (300-350 gm; Simonsen, Gilroy, Calif) were used. Animals were housed in an environmentally controlled room and were given food and water ad libitum. The care of animals complied with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National research Council, Washington, DC, National Academy Press, 1996). All protocols were approved in accordance with the Committee on Animal Research at the University of California, San Francisco.

Rat hindlimb ischemia model. Rats were anesthetized through the inhalation of 1% to 2% isofiurane. All

surgery was performed with a stereomicroscope (Leica) with a magnification of 16 to 25×. Hindlimb ischemia was created by ligation of the left common iliac, external iliac, and femoral arteries. The untreated right hindlimb arteries served as an internal control. This model of ischemia reduces the maximal blood flow in the ischemic gastrocnemius muscle by 36% at 24 days compared with unoperated controls as measured with fluorescent microspheres (data not shown). In this model, no rats had gangrene of the ischemic hindlimb by 40 days. Adenoviral vectors were infused either immediately after the induction of hindlimb ischemia or 10 days after arterial ligation. All animals were weighed and evaluated daily with examination of the hindlimb and paws. Pressure sores, capillary refill, muscle atrophy, clubbing of the toes, and gangrene were recorded. Pressure sores were defined as partial-thickness skin loss on the pads of the paws, which presents clinically as a shallow ulcer. Capillary refill, the length of time required for the paw to return to its normal pink color after 1 to 2 seconds of blanching, was defined as normal or delayed when compared with the contralateral (unoperated) side. Muscle atrophy was defined as a decrease in muscle mass as a percent of total body mass. Gangrene was defined as local soft tissue death involving the foot or hindlimb.

Hindlimb vascular isolation and adenovirus infusion. Hindlimb vascular isolation and intra-arterial administration of adenoviral vectors in ischemic hindlimbs were performed as we have described previously for nonischemic rat hindlimbs.[3] Rats were anesthetized with inhalation of 1% to 2% isoflurane, and a tourniquet was applied at the level of the groin, excluding the femoral nerve. The left femoral vein and its branches were occluded with atraumatic microvascular clamps. The left saphenous artery was isolated and cannulated with a blunt 26-gauge needle in retrograde fashion. A venotomy was made in the saphenous vein, and the limb was infused through the saphenous artery cannula with 10 mL of PBS containing 50 U/mL heparin followed by 5 mL of PBS without heparin. The venotomy was temporarily clamped, and 0.7 mL of PBS or adenovirus (at concentrations ranging from 1×10^8 to 1×10^{10} pfu/mL) was injected through the saphenous artery. After a 30-minute incubation period, the vein clamp was removed, and vector was flushed out of the limb by infusion of 5 mL of PBS. Both the saphenous artery and vein were ligated with 5-0 silk suture, and the clamps were removed. In selected animals, 8 mg/kg of cyclosporine was administered by intraperitoneal injection immediately after ischemia and vascular isolation and continued daily for 7 days. This dose has previously been shown to prolong adenoviral gene expression in vivo.[14] In our laboratory, zero out of five rats developed gangrene after induction of hindlimb ischemia and isolated vascular perfusion with PBS and ligation of the saphenous artery and vein.

An eNOS protein analysis with Western immunoblotting. Gastrocnemius muscles from left (ischemic) and right (control) hindlimbs were harvested, weighed, and homogenized in ice-cold buffer (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.25 mol/L sucrose, and 20 μg/mL each phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, leupeptin, and aprotinin) and centrifuged at 3500 rpm for 30 minutes at 4°C. Supernatant protein levels were determined by standard BCA assay, and 90 μg of each sample was fractionated on 7.5% SDS-PAGE gels and transferred to nylon membranes (Bio-Rad Laboratories, Hercules, Calif). After they were blocked for 1 hour at room temperature with 5% nonfat dry milk and 0.1% Tween 20, membranes were incubated overnight with primary polyclonal rabbit anti-eNOS (1:1000 in milk block) at 4°C (Transduction Laboratories, Lexington, Ky). The membranes were washed in PBS with 0.05% Tween 20 for 30 minutes. The eNOS protein was detected with horseradish peroxidase labeled with donkey and antirabbit immunoglobulin G (IgG) secondary antibody (1:5000 in milk block) (Amersham, Life Science, Inc, Piscataway, NJ) and visualized by chemiluminescence with ECL Plus (Amersham, Life Science Inc).

Determination of nitric oxide synthase activity. Nitric oxide synthase (NOS) activity was determined with a commercial NOS assay (Calbiochem-Novabiochem Corp, San Diego, Calif) that measures the conversion of L-arginine to L-citrulline by NOS. Gastrocnemius muscles were harvested, weighed, and homogenized in ice-cold buffer (25 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA) and centrifuged at 1500 rpm for 5 minutes at 4°C. Ten microliters of the supernatant was added to 40 μL of reaction medium including reaction buffer (50 mmol/L Tris-HCl, pH 7.4, 6 μmol/L tetrahydrobiopterin, 2 μmol/L FAD, 2 μmol/L FMN), 5 μL of 10 mmol/L NADPH, 1 μL of arginine labeled with carbon 14 (1 μCi/μL), 5 μL of 6 mmol/L $CaCl_2$, and 4 μL of H_2O. Identical samples were prepared without $CaCl_2$ to determine the amount of calcium-dependent NOS activity. After an incubation of 30 minutes at 37°C, the reaction was stopped with 400 μL of 50 mmol/L HEPES, pH 5.5, and 5 mmol/L EDTA. We added 100 μL of equilibrated anion exchange resin (Dowex-50) to the reaction sample and centrifuged for 30 seconds at 1500 rpm. ^{14}C-labeled citrulline was quantitated in the supernatant with a scintillation counter. The calcium-dependent NOS activity was calculated by subtracting the NOS activity measured without calcium from the total NOS activity measured with calcium. The results are reported as the amount of labeled citrulline (counts per minute) per milligram of protein in the homogenate.

Tissue preparation and histology. Hamstrings and gastrocnemius muscles were harvested and weighed, and the midsection was cut transversely, embedded in Gum Tragacanth (Sigma) and frozen in precooled (at –70°C) 2-methybutane (Fisher, Pittsburgh, Pa). Serial 10-μm thick frozen sections of the muscles were adhered to poly-L-lysine coated slides, air dried, and fixed in acetone and 1% paraformaldehyde at 4°C for 5 minutes. Muscle sections were stained with hematoxylin and eosin (H&E) 18 hours after vascular isolation or with eNOS 3 days after delayed

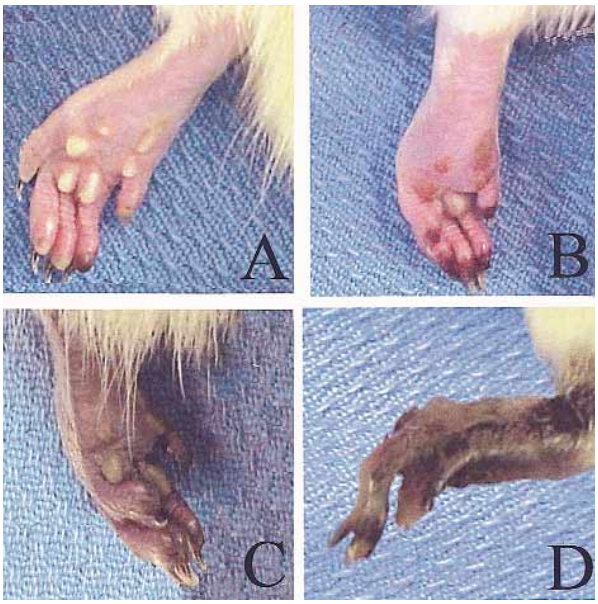

Fig 1. Appearance of ischemic hindlimb after intra-arterial adenoviral gene transfer as a function of viral titer (3-11 days after delivery). **A,** PBS. Note absence of gangrene. **B,** Ad-E1 at 1×10^8 pfu/mL. Note gangrenous toes (occurred in 1 of 3 animals). **C,** Ad-E1 at 1×10^9 pfu/mL. **D,** Ad-E1 at 1×10^{10} pfu/mL.

isolation. Immunohistochemical analysis for eNOS was performed with a mouse IgG monoclonal antibody to rat eNOS (Transduction Laboratories). Goat serum (5%) and 0.2% bovine serum albumin were used for blocking nonspecific binding of protein for 20 minutes. Anti-eNOS antibody (1:250) was incubated at 4°C overnight. Sections were washed in PBS for 5 minutes, and biotinylated goat antimouse IgG was applied for 30 minutes. Sections were again washed in PBS, and an avidin-biotin horseradish peroxidase complex was applied for 30 minutes. Slides were rinsed with PBS, and 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide were applied for 5 minutes and washed with water. Muscle sections were examined for inflammatory cellular infiltrate and edema on H&E and positive staining of eNOS (reddish-brown color) with light microscopy and photographed (40× and 80×).

RESULTS

The effect of adenovirus on acute hindlimb ischemia. Intra-arterial administration of adenoviral vectors at 1×10^9 and 1×10^{10} pfu/mL (AdeNOS and Ad-E1) resulted in gangrene of the entire hindlimb (Fig 1, *C,* and *D*). Within the first 24 to 48 hours all ischemic hindlimbs were slightly cyanotic, dusky, and edematous. The histologic appearance of the muscles after adenoviral administration revealed more inflammatory cellular infiltration and edema than PBS (Fig 2). After 3 days, the control animals (injected with PBS, n = 5) improved in color, and their edema began to resolve. By the seventh postoperative day, pressure sores developed on the pads of the paws. Hindlimbs that were treated with adenoviral vectors became a darker purple after 3 or 4 days. By the 12th day, gangrene (demarcated level of nonviable tissue) developed, and the animals were humanely killed. The administration of adenovirus at a lower titer (1×10^8 pfu/mL) did not appear to cause limb gangrene, and we subsequently performed a complete dose-response experiment to determine if the incidence of gangrene was related to viral titer. The administration of increasing titers of either AdeNOS or Ad-E1 resulted in an increasing incidence of limb gangrene (Fig 3) with no gangrene seen at 1×10^8 pfu/mL, and all animals had gangrene at 1×10^{10} pfu/mL. The occurrence of limb gangrene was sporadic at intermediate titers with some animals having complete necrosis of the hindlimb (Fig 1, *D*) and others, which received the same titer, showing no tissue loss.

The role of adenoviral gene expression in the cause of gangrene. Treating the adenoviral vector with 8-MOP and exposing it to ultraviolet light prevented adenoviral gene expression. This method prevents DNA transcription in the viral particles, including any transgene. The original particle concentration of the stock adenovirus (Ad-E1) was 6×10^{12} part/mL. After treatment, the total particle concentration of the AdMOP/E1 was 3.1×10^{12} part/mL. The plaque assay demonstrated that 8-MOP treatment destroyed the ability of the adenovirus to infect 293 cells, in which the growth of replication-incompetent E1-deleted adenoviral strains is permissive.[15] No plaques were visualized after 2 weeks with an 8-log dilution. However, the stock adenovirus (Ad-E1) had 30 plaques with a 9-log dilution and 3 plaques with a 10-log dilution (3×10^{10} pfu/mL). The resulting titer of the 8-MOP–treated adenovirus ($< 10^8$ pfu/mL) is less than a dose of adenovirus (1×10^8 pfu/mL) not treated with 8-MOP that did not cause gangrene (Figs l, *B,* and 3).

The animals that were treated with AdMOP/E 1 were given an equivalent particle concentration as those that received Ad-E1 or AdeNOS (2×10^{12} part/mL). All three animals that received the AdMOP/E1 also had gangrene by 7 days postoperatively (Fig 4, *D*), demonstrating that it is the adenoviral particle and not gene expression from the adenoviral genome that is responsible for limb gangrene.

Effect of immunosuppression on adenovirus-induced gangrene. Cyclosporine has been shown to prevent immune-mediated reduction in adenoviral gene expression and to decrease the inflammatory response induced by adenovirus.[16] Immediately after ischemia and vascular isolation with intra-arterial injection of Ad-E1 at 1×10^9 pfu/mL (a dose that consistently produced limb gangrene), rats were treated with cyclosporine for 7 days. All three animals that underwent treatment with cyclosporine that began immediately after gene administration and daily thereafter for 7 days had no signs of gangrene by the seventh postoperative day (Fig 4, *C*). There was no evidence of toxicity from the cyclosporine, such as seizures.

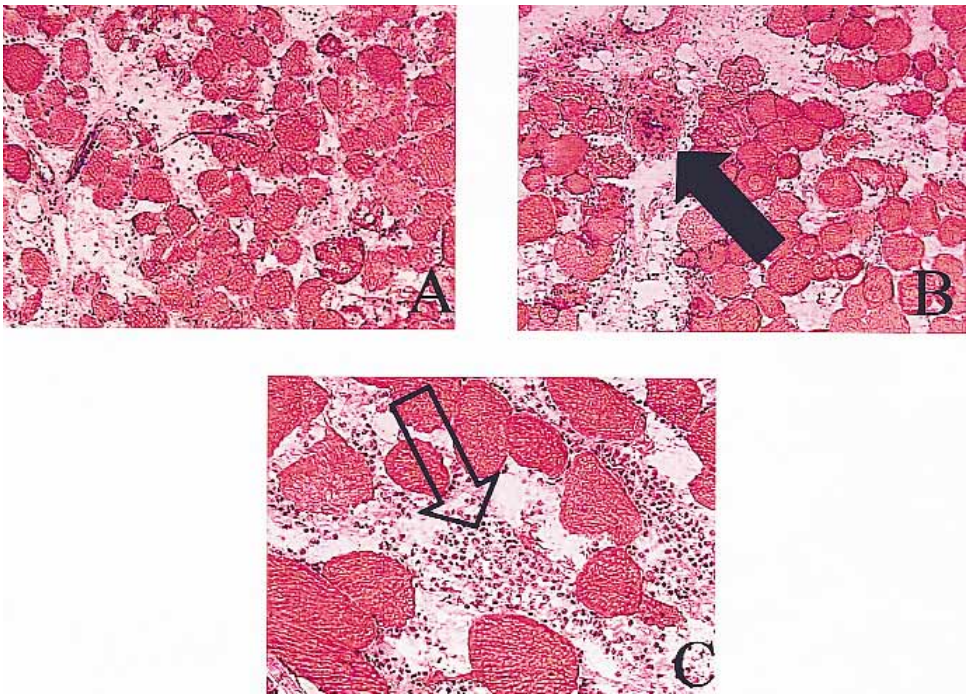

Fig 2. The H&E staining of hamstring muscle harvested 1 day after intra-arterial delivery of PBS **(A)** and AdeNOS **(B)** and in an acutely ischemic hindlimbs **(C)**. Note presence of edema and vasculitis (*black arrow*) in adenoviral-treated muscle 40× **(B)**. Inflammatory cellular infiltration (*open arrow*) was also greater in adenoviral-treated groups 80× **(C)**.

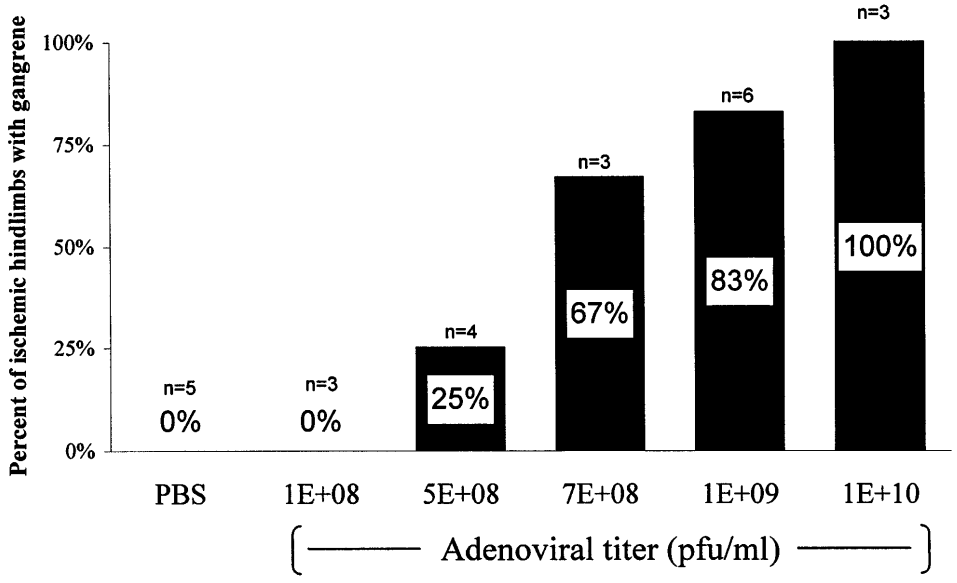

Fig 3. Relationship between PBS or adenoviral titer and incidence of gangrene after intra-arterial administration of adenovirus. Percent of ischemic hindlimbs that developed gangrene after intra-arterial delivery of adenoviral vector at their respective titers (1E + 8 = 1×10^8 pfu/mL).

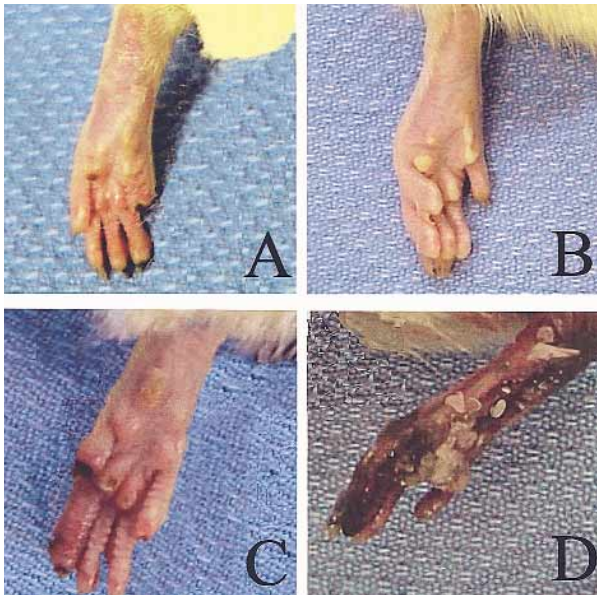

Fig 4. Effect of delayed gene delivery, cyclosporine, and psoralen on development of gangrene after intra-arterial delivery of adenovirus. **A,** 14 days after delayed intra-arterial delivery of PBS. **B,** 6 days after intra-arterial delivery of Ad-E1 at 1×10^9 pfu/mL and treatment with cyclosporine. **C,** 14 days after delayed intra-arterial delivery of Ad-E1 at 1×10^{10} pfu/mL. **D,** 5 days after intra-arterial delivery of psoralen inactivated Ad-E1.

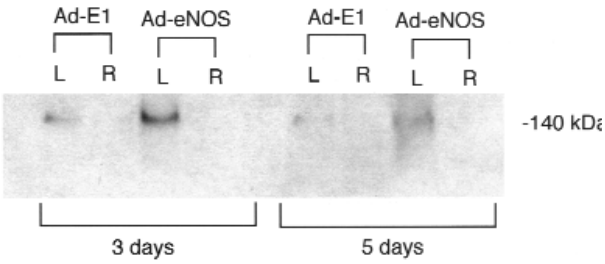

Fig 5. Western blot of gastrocnemius muscle harvested 3 and 5 days after delayed intra-arterial gene delivery in ischemic hindlimb. Band visualized at 140 kd. *Ad-E1*, Gastrocnemius muscle after delivery of Ad-E1; *AdeNOS,* gastrocnemius muscle after delivery of AdeNOS; *L,* left gastrocnemius muscle (ischemic); *R,* right gastrocnemius muscle.

Delayed administration of adenovirus in limb ischemia. We hypothesized that delaying the administration of adenoviral vector until the acute inflammatory response to induction of ischemia had subsided would prevent the development of gangrene. Ten days after ischemia was induced, the adenoviral vector was administered as described above. No gangrene was noted after 14 days in the three animals that received delayed administration of Ad-E1 at 1×10^{10} pfu/mL or in the three rats that received delayed administration of PBS (Fig 4, *A* and *B*). This same dose of adenovirus (1×10^{10} pfu/mL) consistently produced gangrene when administered at the same time that ischemia was induced (Fig 3).

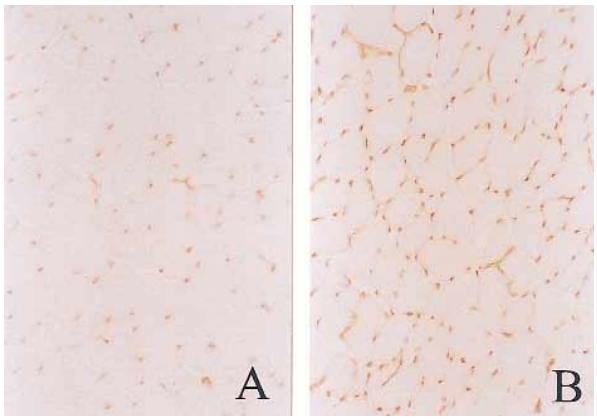

Fig 6. Immunohistochemical staining for eNOS protein in gastrocnemius muscle harvested 3 days after delayed intra-arterial gene delivery in ischemic hindlimb that was transduced with Ad-E1 (A) and AdeNOS (B). Note that positive stain for eNOS protein (reddish-brown staining) is demonstrated to be much stronger in AdeNOS transduced compared with Ad-E1 transduced gastrocnemius muscle(80X).

We then proceeded to evaluate adenoviral gene expression after delayed administration in ischemic hindlimbs. Western blot analysis demonstrated easily detectable gene expression of eNOS protein 3 days and 5 days after delayed administration of AdeNOS at 1×10^{10} pfu/mL (Fig 5), which was not seen in the contralateral (right) limb or in the animals receiving Ad-E 1 adenovirus. NOS activity assay performed on the gastrocnemius muscle of rats 3 days after either AdeNOS or Ad-E1 adenovirus demonstrated a 10-fold increase in NOS activity in the animals receiving AdeNOS virus (444106 ± 39730 cpm/mg vs 174939 ± 49707 cpm/mg protein, calcium-dependent activity, AdeNOS vs Ad-E1, n = 3, Student *t* test, $P < 05$). Immunohistochemical staining of the gastrocnemius muscle 3 days after administration of AdeNOS revealed strong staining for eNOS protein in comparison with the muscle treated with Ad-E1 (Fig 6).

DISCUSSION

We studied the ability of adenoviral vectors to achieve gene transfer into capillary beds of ischemic limbs and examined the effect of adenovirus infusion on limb ischemia. Our major findings were that (1) arterial administration of adenoviral vectors caused gangrene of an ischemic rat hindlimb in a dose-dependent manner, (2) prevention of adenoviral DNA transcription by psoralen failed to prevent the development of gangrene, (3) immunosuppression with cyclosporine prevented the development of gangrene, and (4) delayed administration of AdeNOS by 10 days after the development of ischemia prevented the development of gangrene and achieved elevated expression of eNOS.

This study is the first report of adenoviral gene transfer in acute limb ischemia, and our unanticipated finding of adenoviral-induced gangrene suggests that these vectors should be used with caution in acute limb ischemia. Although adenoviral vectors are highly efficient means by which to achieve gene transfer and hold promise for the treatment of vascular disorders, inflammatory host responses and brevity of transgene expression have limited their use. Adenovirus-mediated gene transfer to large arteries[17,18] and capillaries[3] results in transient expression of biologically significant amounts of transgene products. Exposure of normal arteries to adenovirus, however, has deleterious effects including the development of neointimal hyperplasia, lymphocyte infiltration of the vessel wall, and endothelial cell activation.[7] Furthermore, administration of adenoviral vectors to arteries of hypercholesterolemic rabbits[1] and nonhuman primates[8] accelerates the development of macrophage-rich intimal lesions. We have also extended these findings significantly by demonstrating that increased inflammatory cellular infiltration and edema with adenoviral vectors may exacerbate acute limb ischemia, leading to gangrene and tissue loss. The development of gangrene may be specific to intra-arterial administration of adenoviral vectors, causing a compartment syndrome, and it may be possible that intramuscular injection of adenovirus may not induce a similar response.

Our studies suggest that the exacerbation of acute limb ischemia by adenoviral vectors is mediated by host immune responses targeted toward viral capsid proteins. Although the adenoviral vector may contain other proteins, the capsid proteins comprise approximately 70% of the total adenoviral protein.[11] Also, inhibition of adenoviral DNA transcription by psoralen-inactivation failed to prevent the development of gangrene. Similar reports of inflammatory cellular infiltration of rat salivary glands[19] and murine lungs[20] after exposure to psoralen-inactivated adenovirus support our findings. The use of psoralen excludes the possibility that vector gene expression is responsible for the inflammation seen in our study. Later generations of adenoviral vectors have extensive deletions in the viral genome, which decrease expression of viral genes and have demonstrated decreased inflammation in other in vivo models.[16] Our findings with psoralen suggest that these modified vectors will not prevent development of gangrene because their viral packaging proteins are identical to first-generation adenoviral vectors.

Prevention of gangrene in our model by immunosuppression with cyclosporine confirms that host-immune responses contribute to the deleterious effects observed after administration of adenoviral vectors to acutely ischemic limbs. By itself, acute limb ischemia is characterized by inflammation.[21] The addition of host-inflammatory responses to adenoviral proteins in this setting undoubtedly increases tissue injury, causing gangrene. Adenoviral vectors with modified capsid proteins, nonviral vectors, or immunosuppression treatment may therefore be helpful in preventing the adverse effects of adenoviral gene transfer to ischemic limbs.

Although the administration of adenoviral vectors to acutely ischemic limbs causes gangrene, delaying vector administration for 10 days mitigated this effect. Importantly, we found that delay of vector administration not only prevents gangrene but also leads to significant transgene expression. After the administration of AdeNOS, eNOS protein in the gastrocnemius muscle detected with Western blotting and immunohistochemistry was more abundant than control (Ad-E1)–treated limbs. Greater eNOS protein also resulted in elevated NOS activity within ischemic rat hindlimb muscle. These findings confirm that overexpression of specific transgenes within capillary endothelium and skeletal muscle of ischemic limbs is possible and holds promise for the treatment of critical limb ischemia.

In summary, capillary and skeletal muscle adenoviral gene transfer in the setting of acute limb ischemia elicits a host-inflammatory response that leads to gangrene. This effect was prevented by delayed administration of adenoviral vectors after the development of ischemia or by immunosuppression. This dramatic and possibly clinically significant complication of adenovirus administration in the face of acute ischemia is particularly relevant given the recent focus on potential adverse effects of adenoviral vectors in human gene therapy trials.[22] Circumventing these barriers will allow the development of safe gene therapy approaches for the treatment of critical limb ischemia.

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